Imbalance of regulatory T cells in human autoimmune diseases

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Summary

The breakdown of mechanisms assuring the recognition of self and non-self is a hallmark feature of autoimmune diseases. In the past 10 years, there has been a steadily increasing interest in a subpopulation of regulatory T cells, which exert their suppressive function *in vitro* in a contact-dependent manner and preferentially express high levels of CD25 and forkhead and winged-helix family transcription factor forkhead box P3 (FOXP3) (TREGs). Recent findings of changed prevalences and functional efficiencies indicate that these TREGs play a unique role in autoimmune diseases. Clinical findings in patients with mutated FOXP3 genes and a specific polymorphism in the promotor region of FOXP3 also support the role of FOXP3 as a 'master control gene' in the development and functioning of TREGs. Both altered generation of TREGs and insufficient suppression of inflammation in autoimmune diseases are considered to be crucial for the initiation and perpetuation of disease. TREG-related somatic cell therapy is considered as an intriguing new intervention to approach autoimmune diseases.

Keywords: autoimmune disease; FOXP3; regulatory T lymphocyte; somatic cell therapy; suppressor cells

Introduction

The breakdown of mechanisms assuring recognition of self and non-self by the immune system is a hallmark feature of autoimmune diseases. The primary mechanism leading to self-tolerance has recently been termed as 'recessive tolerance', which is induced by the thymic deletion of autoreactive T cells.[1] However, thymic selection is incomplete, and self-reactive cells occur, even in healthy individuals. On the other hand, 'dominant tolerance' is an additional mechanism for maintaining peripheral self, which is mediated by regulatory T cells actively modulating immune responses.[2]

In the past 10 years, there has been a steadily increasing interest in regulatory T cells. The recognition of regulatory T cells, originally termed suppressor T cells, resulted from experiments performed in the 1960s and 1970s which described the induction of suppressor T cells capable of down-regulating antigen-specific T-cell responses.[3] Several subtypes of regulatory T cells have been defined since then, each with a distinct phenotype, cytokine-production profile and mechanism of action for suppressing immune responses. Some of these regulatory T cells are $CD8^{+4}$ others are $CD4^{+}$. In the $CD4^{+}$ regulatory T-cell compartment, detailed analysis led to identification of the interleukin (IL)-10-producing T-regulatory cell type 1 (Tr1),[5] transforming growth factor-β (TGF-β)-secreting T-helper cell type 3 (Th3)[6] and a subpopulation of 'naturally occurring' regulatory T cells that exert their suppressive function *in vitro* in a contact-dependent manner and preferentially express high levels of CD25 and the forkhead and winged-helix family transcription factor

Abbreviations: AIRE, autoimmune regulator; APECED, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; FOXP3, forkhead and winged-helix family transcription factor forkhead box P3; GITR, glucocorticoid-induced tumor necrosis factor receptor; HCV, hepatitis C virus; HLH, haemophagocytic lymphohistiocytosis; IL, interleukin; IPEX, immunodysregulation, polyendocrinopathy, enteropathy X-linked; NFAT, nuclear factor of activated T cell; PD-L1, programmed cell death-ligand 1; TCR, T-cell receptor; TGF-β, transforming growth factor-β; Th3, T helper cell type 3; TNF-α, tumour necrosis factor-α; Tr1, T regulatory cell type 1; Trec, T-cell receptor excision circles.

forkhead box P3 (FOXP3) (TREGs).[7] In this review we summarize recent findings about the unique role of TREGs in autoimmune diseases.

Surface characterization of human TREGs

The definition of human TREGs is still under discussion and no definite surface marker is currently available. The high constitutive surface expression of the IL-2 receptor alpha chain (CD25) is generally considered as a characteristic feature of the majority of human TREGs, and regulatory activity is enriched in $CD4^+$ T cells expressing the highest levels of CD25 ($CD4^+$ $CD25^{hi}$ T cells).[8–12] CD25 expression on recently activated non-regulatory T cells is usually lower than on peripheral TREGs. However, there is a lack of consensus on a cut-off defining high expression of CD25 in flow cytometry analysis.

A considerable number of other surface markers have been reported to be expressed on human $CD4^+$ $CD25^{hi}$ T cells, including cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), CD62 ligand (CD62L, also known as L-selectin), CD134 (OX40), glucocorticoid-induced tumor necrosis factor receptor (GITR), membrane-bound TGF-β, CD95, programmed cell death-ligand 1 (PD-L1) and $\alpha_4\beta_7/\alpha_4\beta_1$ integrin.[8,9,11,13–21] Upon activation of T cells, and independently of their regulatory capacity, most of these markers become up-regulated and have therefore only limited specificity to identify TREGs.[22]

Transcription factor FOXP3 and TREGs

Intracellular expression of FOXP3 is currently considered as the most specific marker for human TREGs.[23–25] Human *FOXP3* is localized on the X chromosome encoding 'scurfin', which binds to the IL-2 promotor and the granulocyte–macrophage colony-stimulating factor enhancer near the nuclear factor of activated T cell (NFAT) sites. FOXP3 represses these genes, thus reducing IL-2 production by $CD4^+$ T cells.

From the clinical perspective, a mutation of this transcription factor is strongly linked to immune dysregulation. Patients with a mutated *FOXP3* gene encounter autoimmune polyendocrinopathy (especially type 1 diabetes mellitus and hypothyroidism) and enteropathy [summarized as 'immunodysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome'].[26] Furthermore, a specific polymorphism in the promotor region of *FOXP3* is associated with autoimmune diabetes, suggesting that *FOXP3* variants may be linked to the susceptibility to autoimmune diseases.[27] In mice, a deficiency of TREGs in *FOXP3* mutants was observed, together with an autoimmune syndrome similar to IPEX.[25] *In vitro*, retroviral expression of *FOXP3* in human T cells turned otherwise non-regulatory naive T cells into a TREG-like phenotype with the surface expression of CD25 and suppressive activity.[28,29] Murine T cells retrovirally expressing *FOXP3* even prevented IPEX-like disease in *FOXP3* mutant mice.[30]

These studies have all supported the role of *FOXP3* as a 'master control gene' in the development and functioning of TREGs.[25] Surprisingly, the importance of FOXP3 has recently been challenged by *in vitro* experiments showing that FOXP3 mRNA transcripts and protein are also expressed in activated non-regulatory human T cells. In contrast to previous observations, FOXP3 induction was not associated with suppressive activity in these activated T cells.[31,32] However, it cannot be excluded that this finding depends on the experimental setting, as TREG suppression can be over-ruled by strong activation, for example, in the presence of high doses of cross-linked anti-CD3 immunoglobulin and IL-2.[10,21,33]

Levels of TREGs in autoimmune diseases

Circulating $CD4^+$ $CD25^{hi}$ T cells

Although several efforts have been made to combine different surface markers for a more specific characterization of TREGs,[34,35] gating on $CD4^+$ $CD25^{hi}$ T cells is usually preferred to define TREGs, as mentioned above.

The level of circulating $CD4^+$ $CD25^{hi}$ T cells out of the $CD4^+$ T-cell pool in healthy humans ranges from 0·6% to 7·9% (Table 1). In patients with autoimmune diseases, reduced levels of circulating $CD4^+$ $CD25^{hi}$ T cells were described, specifically in individuals with juvenile idiopathic arthritis,[9,17] psoriatic arthritis,[17] hepatitis C virus (HCV)-associated mixed cryoglobulinaemia,[36] autoimmune liver disease,[37] systemic lupus erythematosus[20,38] and Kawasaki disease.[39] Lower levels of circulating $CD4^+$ $CD25^{hi}$ T cells also correlate with a higher disease activity or poorer prognosis.[9,36–39] It has been proposed that the reduced levels may be caused by the impaired proliferation of peripheral $CD4^+$ $CD25^{hi}$ T cells, as observed *in vitro* (Table 2).[21,37] Thereby, the balance between pro-inflammatory and regulatory T cells would be disturbed, leading to the breakdown of self-tolerance. However, further studies are required to confirm these results *in vivo* and to exclude any bias of these experiments owing to contamination with anergic effector T cells expressing high levels of CD25.[22]

In contrast, there was no significant difference in the pool of $CD4^+$ $CD25^{hi}$ T cells between healthy controls and patients with spondyloarthritis,[17] multiple sclerosis,[19,21] or immune-mediated type 1 diabetes mellitus.[16,40] An increased prevalence of circulating $CD4^+$ $CD25^{hi}$ T cells was even observed in patients with primary Sjögren syndrome,[41] and conflicting data have been reported for myasthenia gravis[42,43] and rheumatoid arthritis,[11,17,18,20,44–46] with decreased or similar levels of peripheral $CD4^+$ $CD25^{hi}$ T cells compared with healthy

Table 1. Prevalences of $CD4^+$ $CD25^{hi}$ T cells in peripheral blood (PB) and sites of inflammation of patients with autoimmune diseases and healthy controls

	Reference	Number of patients (controls)	Prevalence† of $CD4^+$ $CD25^{hi}$ T cells in PB of patients (controls)		Prevalence† of $CD4^+$ $CD25^{hi}$ T cells at sites of inflammation		Prevalence† of $CD4^+$ $CD25^{hi}$ T cells versus clinical and serological parameters
Juvenile idiopathic arthritis	9	60 (34)	1·2 (1·6)	↓***	6·2	↑***	Persistent
		34 (34)	0·5 (1·6)	↓***	5·2	↑***	Extended
Juvenile idiopathic arthritis	17	21 (29)	0·4 (1·2)	↓***	3·7	↑***	None
Rheumatoid arthritis	17	135 (29)	0·7 (1·2)	↓***	2·3	↑***	None
Rheumatoid arthritis	18	79 (67)	1·5 (1·1)	≈	7	↑***	$Corr_{coeff}$ (with ESR) = 0·357**
Rheumatoid arthritis	11	27 (7)	0·7 (1·1)	≈	7·1	↑**	
Rheumatoid arthritis	20	52 (50)	1·2 (3·7)	↓*			
Rheumatoid arthritis	44	33 (8)	3 (3)	≈			$Corr_{coeff}$ (with CRP) = −0·528***
Rheumatoid arthritis	45	10 (9)		≈		↑	
Rheumatoid arthritis	46	8 (5)	(4·1)	≈			
Spondyloarthritis	17	10 (29)	1·2 (1·2)	≈	3·4	↑***	None
Psoriatic arthritis	17	26 (29)	0·6 (1·2)	↓*	2·6	↑***	None
HCV mixed cryoglobulinaemia	36	26 (5)	7·4 (7·9)	≈			Asymptomatic
		22 (5)	2·6 (7·9)	↓**			Symptomatic
Multiple sclerosis	19	60 (15)	0·9 (0·6)	≈			
Multiple sclerosis	21	15 (21)	1·2 (1·4)	≈			
Autoimmune liver disease	37	30 (18)	2·5 (6·8)	↓***			Active $Corr_{coeff}$ (with LKM titre) = −0·879** $Corr_{coeff}$ (with anti-SLA titre) = −0·600*
		28 (18)	4·2 (6·8)	↓*			Remission
Systemic lupus erythematodes	20	94 (50)	1·8 (3·7)	↓*			None
Systemic lupus erythematodes	38	10 (10)	0·9 (2·6)	↓*			Active
		20 (10)	1·6 (2·6)				Remission
Immune-mediated diabetes	16	21 (15)		≈			Recent onset
Immune-mediated diabetes	40	17 (19)	1·0 (1·0)	≈			Longstanding
Myasthenia gravis	42		1·2 (1·2)	≈			
Myasthenia gravis	43	38 (17)		↓*			
Primary Sjögren syndrome	41	21 (21)	8·5 (4·1)	↑*			None
Kawasaki disease	39			↓			Active Deffervescence
Inflammatory bowel disease	10	49 (54)		≈		↑*	

The first column indicates the disease and the third the number of patients and healthy controls (controls) enrolled in each study. The fourth column shows the prevalence of PB $CD4^+$ $CD25^{hi}$ T cells from patients compared with controls (in parenthesis) and the fifth column the prevalence of $CD4^+$ $CD25^{hi}$ T cells at sites of inflammation compared with PB of patients. (↑) Indicates an increased, (↓) a decreased, and (≈) an equal prevalence of $CD4^+$ $CD25^{hi}$ T cells, respectively. The sixth column shows correlations between prevalences of $CD4^+$ $CD25^{hi}$ T cells in PB and clinical/serological parameters.

'None' indicates that experiments detected no correlation. Significance levels are shown as: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; and $***P < 0{\cdot}001$.

Anti-SLA, antibodies to soluble liver antigen; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; HCV, hepatitis C virus; LKM-1, liver kidney microsomal antibody type-1.

†Expressed as a percentage relative to total $CD4^+$ T cells.

controls. Thus, a reduced prevalence of circulating TREGs is not a general finding in all patients with autoimmune diseases and does not also necessarily reflect the actual situation at sites of inflammation.

$CD4^+$ $CD25^{hi}$ T cells at inflammatory sites

There is agreement among many investigators of increased recruitment of $CD4^+$ $CD25^{hi}$ T cells at sites

Table 2. Functional efficiency (proliferation, suppression of proliferation and suppression of cytokine production) of $CD4^+$ $CD25^{hi}$ T cells from patients with autoimmune diseases

	Reference		Proliferation	Suppression of proliferation	Suppression of cytokine production	Prevalence/function of TREGs versus treatment (agents)
Juvenile idiopathic arthritis	9	Persistent		+		None (MTX)
		Extended		+		
Juvenile idiopathic arthritis	17			+	+	None (MTX, CLQ, CS, CS injections)
Rheumatoid arthritis	17			+	+	None (MTX, CLQ, CS, CS injections)
Rheumatoid arthritis	18		+	≈	+	None (MTX, CS, TNF-α blocking)
Rheumatoid arthritis	11			+		
Rheumatoid arthritis	44		+	≈	↓	Prevalence↑/function↑ (TNF-α blocking)
Rheumatoid arthritis	45			+		
Rheumatoid arthritis	46			↓		Prevalence↑/function† ↑ (TNF-α blocking)
Spondyloarthritis	17			+	+	None (MTX, CLQ, CS, CS injections)
Psoriatic arthritis	17			+	+	None (MTX, CLQ, CS, CS injections)
HCV mixed cryoglobulinaemia	36	Symptomatic	+	↓		
Multiple sclerosis	21		↓***	↓*	↓	
Polyglandular syndrome type II	64		+	↓**		
Autoimmune liver disease	37		↓*		≈	
Immune-mediated diabetes	16		≈	↓***	↓*	
Immune-mediated diabetes	40			≈	≈	
Myasthenia gravis	42		↑	↓		
Myasthenia gravis	43					Prevalence↑ (CS, AZA)
Primary Sjögren syndrome	41			+		None (MTX, CS)
Inflammatory bowel disease	10			≈		None (CS, SSZ, AZA)

(↑) Indicates an increased (↓) indicates a decreased and (≈) indicates an equal functional capacity of circulating $CD4^+$ $CD25^{hi}$ T cells from patients compared with controls.

(+) Indicates the occurrence of the corresponding function and that it was not compared between patients and controls.

The last column shows correlations between prevalences and function of $CD4^+$ $CD25^{hi}$ T cells and therapeutic interventions.

'None' indicates that experiments detected no correlation.

Significance levels are shown as follows: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; and $***P < 0{\cdot}001$.

AZA, azathioprine; CS, corticosteroids; CLQ, chloroquine; HCV, hepatitis C virus; MTX, methotrexate; SSZ, sulfasalazine; TNF-α, tumour necrosis factor-α.

†Transient effect observed on day 15, but not on day 180 after therapy.

of inflammation compared with peripheral blood (Table 1).[9–11,17,18,45] Such enrichment has been shown to be stable over time and independent of clinical and laboratory parameters, disease duration and therapeutic interventions, including intra-articular corticosteroid applications or the use of tumour necrosis factor-α (TNF-α) blocking agents (Table 2). These $CD4^+$ $CD25^{hi}$ T cells display an activated phenotype expressing high levels of CTLA-4 and low levels of CD62L. Functional assays revealed an increased suppressive potency, indicating $CD4^+$ $CD25^{hi}$ T cells to be crucial players in the modulation of local immune responses.[9,10,18] This is in line with the observation that $CD4^+$ $CD25^{hi}$ T cells accumulate in inflammatory lesions during infections, preventing collateral tissue damage.[47] However, in autoimmune diseases, even the enrichment of $CD4^+$ $CD25^{hi}$ T cells with increased suppressive potency at sites of inflammation is insufficient to interrupt inflammation, thus indicating an imbalance between pro-inflammatory and regulatory T cells, as outlined below.[48]

Different developmental stages of TREGs and TREG homing

The observation that murine TREGs expressing FOXP3 constitute several phenotypically and functionally distinct subsets,[47,49,50] led to the concept of developmental stages of TREGs. In brief, one subgroup of $CD4^+$ $CD25^+$ TREGs expresses high surface levels of CD62L and the chemokine receptor CCR7, and preferentially homes to antigen-draining lymph nodes (similarly to naive T cells), where they efficiently inhibit induction of inflammatory reactions.[49–51]

Another subgroup of TREGs, expressing $\alpha_E\beta_7$ integrin, primarily traffics into non-lymphogenic tissues and sites of inflammation directed to down-modulate local immune reactions at these sites. These TREGs are either $CD25^+$ or $CD25^-$ and have undergone repetitive cell divisions, as indicated by a low number of T-cell-receptor excision circles.[49,50] As TREGs isolated from neonatant mice lack α_E integrin expression and acquire this marker with aging,[52] it has been proposed that during the course of antigenic stimulation, TREGs might change their phenotype and their migratory activity from naive-like into memory/effector-like TREGs to exit into (inflamed) tissues.[47] In accordance with this mouse-derived model, the occurrence of naive-like ($CD45RA^+$ $CD62L^{hi}$ $CCR7^{hi}$) and antigen-experienced TREGs ($CD45RO^+$ $CD62L^{int}$ $CCR7^{int}$) has recently been shown in healthy humans.[53]

$CD4^+$ $FOXP3^+$ cell populations with low or no CD25 expression also exist in human peripheral blood, non-lymphogenic tissues and at sites of inflammation. Indeed, more than 30% of peripheral blood $CD4^+$ T cells with intermediate levels of CD25, and even 3·6% of $CD4^+$ $CD25^-$ T cells, are positive for FOXP3 in humans and may thus reflect different stages of TREG development, similar to that of the murine system.[9,10,54] According to this concept, reduced levels of circulating $CD4^+$ $CD25^{hi}$ T cells may not necessarily reflect a deficit of TREGs, but rather indicate an increased shift of TREGs from the $CD25^{hi}$ naive-like into the $CD25^{-/low}$ memory/effector phenotype associated with an enhanced traffic towards inflamed tissue, which may, in fact, correlate with disease activity (Table 1). As far as autoimmune diseases with normal or increased prevalences of circulating $CD4^+$ $CD25^{hi}$ T cells are concerned, we can only speculate that $FOXP3^-$ anergic effector T cells (which develop during the course of autoimmune diseases), or other regulatory T cells (such as Tr1), may express high levels of CD25.[22] The presence of such anergic effector T cells might also explain the observation that patients with multiple sclerosis have normal levels of circulating $CD4^+$ $CD25^{hi}$ T cells, although the *in vitro* proliferation of $CD4^+$ $CD25^{hi}$ T cells is reduced.[21]

The limitations of this concept are that the expression of FOXP3 may be less specific for human than for murine TREGs,[32] and the regulatory activity of human $CD4^+$ $CD25^-$ $FOXP3^+$ T cells has not, to date, been determined at the single cell level. Such investigations of FOXP3 or alternative specific markers for the identification of human TREGs are still needed to enable future studies, which address more specifically the relationship between TREGs in peripheral blood and at sites of inflammation, to be undertaken.

Suppressive mechanisms of TREGs

The mechanisms used by TREGs to suppress immune responses are still unresolved, and current hypotheses have been summarized in a number of reviews.[25,47,55] In brief, inhibition is contact dependent, and *trans*-well assays and supernatants of TREGs revealed no suppressive effects. After activation, human TREGs may directly kill activated $CD4^+$ and $CD8^+$ T cells in a perforin- or granzyme-dependent manner *in vitro*.[56] Although evidence for such TREG-mediated cytotoxicity is lacking *in vivo*, the observation of patients with mutations in the *perforin* gene and who suffer from haemophagocytic lymphohistiocytosis (HLH) indicates a critical involvement of perforin in the regulation of immune responses.[57] These patients have an overactive immune system, and immune responses to infections are not down-regulated after the cessation of an infection. Untreated HLH patients then develop end-organ damage from lymphocyte infiltration and macrophage activation. *Perforin*-deficient mice develop a disease similar to HLH after exposure to viruses.[58] *Perforin* polymorphisms may thus predispose to a prolonged activation of the immune system during infections, which could provoke the breakdown of tolerance (see below). Another effector mechanism of suppression has been proposed with reverse signalling through cross-linking B7 (CD80 and CD86) on the cell surface of antigen-presenting cells or activated T cells. This signalling is mediated by CTLA-4 expressed on TREGs.[59] However, as murine TREGs with target deletion of genes encoding CTLA-4 are suppressive *in vitro*, a non-redundant role of CTLA-4 in the suppressive process is unlikely.[60]

Whether cytokines modulate the TREG-mediated suppression of immune responses is also unclear. Both TGF-β and IL-10 have been linked to this effect in murine colitis and type 1 diabetes, although *in vitro* blockade of TGF-β or IL-10 does not totally abrogate suppression.[61–63]

Insufficient suppression of inflammation in autoimmunity

Obviously, TREGs still fail to totally suppress inflammation in patients with autoimmune diseases, despite the local enrichment of TREGs and enhanced suppressive activity *in vitro* (as mentioned above), thus supporting the concept of a profound imbalance between pro-inflammatory and regulatory T cells.[48] It has been proposed that one underlying cause for this insufficiency may be a reduced suppressive capacity of TREGs, as observed in *in vitro* assays comparing $CD4^+$ $CD25^{hi}$ T cells from patients with HCV-associated mixed cryoglobulinaemia,[36] multiple sclerosis,[21] polyglandular syndrome type II,[64] myasthenia gravis[42] or rheumatoid arthritis with $CD4^+$ $CD25^{hi}$ T cells from healthy controls[44] (Table 2). However, as Shevach points out, these *in vitro* experiments could be influenced by contamination with activated non-regulatory T cells and Tr1 cells.[22] It is further conceivable that the most potent TREGs are not in the

peripheral blood but exert their suppressive function in the target organ. Leipe *et al.* recently argued in favour of a possible inhibition of TREGs by pro-inflammatory cytokines from the synovial fluids in rheumatoid arthritis.[65,66] IL-7 and IL-15 have been detected in the synovial fluid of patients with juvenile idiopathic arthritis, which strongly reduced the activity of TREGs *in vitro*.[34]

Alternatively, responder T cells may show a decreased susceptibility to TREG-mediated suppression. $CD4^+$ $CD25^-$ T cells from synovial fluid are in fact more difficult to suppress than $CD4^+$ $CD25^-$ T cells from peripheral blood of rheumatoid arthritis patients.[18] For example, IL-6 is increased at sites of inflammation and known to enhance the resistance of $CD4^+$ $CD25^-$ T cells to the suppressive effects of TREGs *in vitro*.[66] Permanent activation of T cells through constitutively and aberrantly expressed costimulatory molecules such as B7.1, B7.2 and MICA, along with T-cell receptor (TCR) stimulation, may perpetuate chronic inflammation despite the presence of TREGs. Stimulation of $CD4^+$ T cells with GITR ligand also results in resistance to TREG-mediated suppression; all of these collectively support the idea of an active inhibition of TREG function at inflammatory sites.[33,67,68]

Generation of TREGs and maintaining the pool of TREGs

Thymic generation of TREGs

TREGs are generated in the thymus, during a positive selection process, by high-affinity interactions of the TCR to cortically expressed host antigens.[69] As this process is paralleled by the depletion of non-regulatory autoreactive T cells expressing the same TCRs, other mechanisms, which are independent of the avidity of the TCR, are suggested to be involved in the development of TREGs.[24,69,70] These mechanisms may include costimulatory molecules out of the B7 or TNF family, such as CD28, PD-1 or CD40L,[55,71] as well as cytokines including IL-2, TNF-α or TGF.[72,73] Depending on these additional signals, thymocytes are then either negatively selected or induce a genetic program for TREGs, including up-regulation of FOXP3 and CD25.[24]

A deficient thymic function in athymic mice causes an impaired generation of TREGs, leading to type 1 diabetes mellitus, thyroiditis, gastritis and systemic wasting disease[7]. Furthermore, *CD28*- and *B7*-deficient mice, or mice with a knockout defect for the *IL-2* gene, show a profound decrease in the number of thymus-derived TREGs. This may be at least partly explained by a 'skew' of the local signals, although the exact function of B7/CD28 and IL-2 for thymic development of TREGs is unknown.[71,74] Thus, an intact thymus and cytokine environment play a crucial role in the development of murine TREGs and the maintenance of dominant tolerance.

In humans, several clinical observations have supported a link between reduced thymic function, with impaired TREG generation, and the induction of autoimmune diseases.

(1) Children with thymic hypoplasia as a result of the *22q.2* deletion syndrome display an impaired TREG generation and have an increased risk of developing an autoimmune disorder.[75]
(2) Patients with a mutation of the *transcription factor autoimmune regulator (AIRE)* have a defective expression of tissue-specific self-antigens in thymus, leading to autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED).[76] In mice, *AIRE* deficiency is associated with a reduced generation of TREGs, which may hold true also for humans.[77]
(3) Reduction of thymic activity has been reported in patients with multiple sclerosis[78] and rheumatoid arthritis,[79] who show a reduced number of T-cell receptor excision circles (Trec), indicating a reduced thymic output.[80,81] The number of Trecs exponentially declines also with aging, and reduction of Trecs in young patients with autoimmune diseases indicates therefore an 'early aging' of the thymus. As TREG function declines with thymic senescence,[82] it is conceivable that the induction of TREGs in the 'early aged' thymus of patients with autoimmune diseases is less efficient, and non-regulatory T cells bearing an autoreactive TCR may escape the thymic selection process more frequently.[83]

Besides, a skew of thymic signals and cytokines may induce the breakdown of self-tolerance. In the thymus of patients with myasthenia gravis, for example, IL-6-producing cells have been detected, and IL-6 abrogates the suppression of human TREGs, as mentioned above.[66,84] Likewise, blockade of TNF-α in patients with rheumatoid arthritis restored the suppressive function of TREGs.[44,46] Although the underlying mechanism for this phenomenon is unclear, it has been suggested that the modification of TNF-α in the thymic cytokine environment might restore TREG generation, rather than TNF-α acting directly on peripheral TREGs.

Peripheral induction of human TREGs

Processes that maintain the size and composition of the peripheral TREG pool include not only the expansion and survival of thymus-derived TREGs, but may also involve the conversion of naive non-regulatory $CD4^+$ $CD25^-$ T cells into TREGs.[85,86] The signals that facilitate or direct peripheral TREG formation remain elusive, but may involve costimulatory molecules such as CTLA-4, cytokines such as TGF-β, and dendritic cells.[25,87] In mice, $CD4^+$ $CD25^-$ $FOXP3^-$ T cells showed *de novo* induction of FOXP3 after antigenic stimulation in the

presence of TGF-β.[86] These newly formed TREGs were capable of suppressing non-regulatory T cells. In humans, however, the preferential expansion of a small population of FOXP3-expressing $CD4^+$ $CD25^-$ T cells after activation in the presence of TGF-β cannot be excluded.[1,88] Further studies are required to clarify whether FOXP3 expression relates directly to the thymic origin of TREGs or to TREG function possibly induced in peripheral naive T cells.[22]

Imbalanced immune homeostasis and autoimmunity

Normal maintenance of the homeostatic equilibrium is achieved through thymic T-cell generation with subsequent development of peripheral T cells and cell death. Under conditions of an extremely disturbed immune system, such as lymphopenia or acute depletion of lymphocytes, T cells undergo peripheral proliferation in the absence of foreign antigen stimulation and can restore the size of the peripheral T-cell compartment independently of the thymic output of naive T cells.[89] As lymphopenic states are common throughout life, for example, during viral infections, lymphopenia-induced proliferation may be the primary mechanism to restore the T-cell pool in aged individuals with reduced thymic function.[90] Although mechanisms that regulate this proliferation are still under investigation, it became apparent that the proliferative capacity of individual T cells correlates with their avidity for self-ligands.[91] Thus, lymphopenia-induced proliferation has the potential to skew the TCR repertoire towards greater self-reactivity.[90] During the course of lymphopenia-induced proliferation, T cells can acquire effector functions, which may explain a long-recognized association of lymphopenia with autoimmune diseases, including Sjögren syndrome, rheumatoid arthritis, systemic lupus erythematodes, polymyositis/dermatomyositis and Crohn's disease. Indeed, in patients with rheumatoid arthritis, a contraction of TCR diversity and oligoclonal T-cell expansion further support this concept.[79,92–96]

Because of the infrequent occurrence of autoimmune diseases, even in people with reduced thymic function and among cancer patients with severe lymphopenia caused by chemotherapy or irradiation, peripheral mechanisms of tolerance have to compensate for the increased autoreactivity of the T-cell pool in healthy individuals. These mechanisms are still not completely understood, but may include the restoration of the TREG pool from a peripheral reservoir[97] and the induction of TREGs from non-regulatory T cells.[98] Moreover, self-antigen-driven proliferation of TREGs during lymphopenia results in the competition with non-regulatory T cells for limited resources such as IL-2 or immunological space, and may thus restrain lymphopenia-induced proliferation of non-regulatory T cells.[99,100]

Breakdown of tolerance by environmental factors

Depletion of $CD25^+$ cells in mice eliminates a high proportion of circulating TREGs and activated T cells with severe lymphopenia, but is not sufficient to induce autoimmunity without administration of strong adjuvants. This observation supports the multifactorial pathogenesis of autoimmunity.[101] Environmental factors, such as microbial infections or drug metabolism, have long been suspected to trigger the onset of autoimmune diseases by antigen-specific and non-specific effects.

Molecular mimicry is one possible mechanism used by microbes to break immune tolerance.[102] The underlying concept is that infectious agents share one or more epitopes with various self-components. An alternative hypothesis is that infectious agents cause bystander activation of immune cells with autoaggressive potential. Thus, infections cause transient lymphopenia and organ damage with a release of autoantigens, favouring the proliferation of T cells bearing an autoreactive TCR.[103] In addition, they provoke the presentation of self-antigens, together with cytokines and costimulatory molecules, as a 'danger' signal.[104] These danger signals are important for inducing an effective immune response against microbes by activating not only naive T cells but also attenuating TREG-mediated suppression.[33,103,105] In the event of disturbed tolerance mechanisms with impaired TREG generation and bias of non-regulatory T cells towards autoreactivity, infections may initiate or even trigger 'pre-existing' autoimmunity. Genetic polymorphisms of molecules influencing TREG generation or activation, such as IL-2, CTLA-4 or CD28,[106,107] the timing of infection and the magnitude of inflammation may be additional factors involved in the exacerbation of autoimmunity.[105]

TREG-mediated therapeutic approaches for the future

Strategies to support TREGs in autoimmune diseases are considered an intriguing new approach for using to suppress the inflammatory process, by manipulating both the function and number of TREGs. It is believed that protocols for such manipulation have the therapeutic potential to induce tolerance in patients with autoimmune diseases, because in mice with collagen-induced arthritis, depletion of TREGs caused rapid progression, but early joint damage could be reversed by the transfer of isolated and *ex vivo*-proliferated TREGs.[108] Other animal models of autoimmunity show similar results.[109,110] Monoclonally expanded TREGs, which specifically target autoantigens, may even provide more efficient suppression,[111] and protocols for the *in vitro* expansion of human TREGs are already available.[112]

Alternatively, transfection of nonregulatory T cells with *FOXP3* could generate antigen-specific TREGs with

increased suppressive activity that target sites of inflammation.[28–30] Transfection of polyclonally expanded TREGs with genes encoding TCR-α and -β chains, or genes encoding a chimeric antigen-MHC-CD3-ζ molecule might also result in significant numbers of potent, highly directed, antigen-specific TREGs.[113]

Other approaches to increase TREG function or numbers without *ex vivo* manipulation include administration of cytokines (such as TGF-β) that favour TREG activity and survival. TGF-β may also convert non-regulatory T cells into TREGs[86] and *TGF-β* gene therapy was found to induce TREGs in syngenic islet transplanted non-obese diabetic mice, blocking islet destructive autoimmunity.[114] Tolerance can also be induced by short-term treatment with monoclonal antibodies against costimulatory molecules, adhesion molecules or the TCR complex.[115–117] Achievement of tolerance, for example, has been described in patients with new-onset type 1 diabetes mellitus using hOKT3, the FcR non-binding anti-CD3 monoclonal antibody (phase I/II trial).[118] With this approach, activation and clonal expansion of naive T cells was prevented by a single 14-day course of hOKT3. Notably, insulinitis dramatically improved with subsequent insulin production and no further need of immune suppression for longer than 1 year. A subpopulation of circulating T cells occurred with suppressive function *in vitro* and with a similar phenotype to TREGs. The same T-cell population was observed later in patients with human islet transplantation treated with hOKT3.[119]

There are, however, technical difficulties regarding reliable identification of TREGs and the generation of sufficient numbers of TREGs for therapeutic purposes.[120] T cells with retroviral expression of *FOXP3* have recently been shown to be less suppressive than freshly isolated $CD4^+$ $CD25^+$ TREGs[121] and may therefore be insufficient to down-regulate autoimmunity in humans. Concerns also exist about the uncontrolled proliferation of TREGs and/or development of unforeseen functional activities, such as differentiation into effector T cells. In the event of a possible underlying genetic defect of TREG functions, stimulating more cells with the same defect may not be an effective approach. Besides, as in most autoimmune diseases, the causative antigen to perform monoclonal expansion of TREGs is unknown, therapy would depend on questionable bystander suppression. Large numbers of TREGs may also increase the risk of developing cancer or affect the immune response against acute and chronic infections.[120,122,123]

In view of the potential dangers associated with the manipulation of TREGs, an alternative approach would be to develop strategies for the prevention of progressive thymic failure; this would preserve an optimal diversity of the TCR repertoire and the generation of thymus-derived TREGs and also avoid the potential undesirable side-effects associated with protocols for manipulating TREGs. Current treatment attempts include the administration of growth hormone or IL-7, which has been found to increase thymus size and cellularity.[124,125] How promising this approach is, and whether its clinical implementation will be devoid of severe adverse events, are issues that only future research can clarify.

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